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(54) Title: RETROVIRAL VECTORS CAPABLE OF TRANSDUCING NON-DIVIDING CELLS

(57) Abstract

In accordance with the present invention, methods have been developed to modify retroviral vectors derived from viruses which are not known to be pathogenic in humans (e.g., MLV), so that such vectors are rendered capable of transducing heterologous sequences into non-dividing cells. Thus, it has been discovered that retroviruses can be rendered capable of infecting non-dividing cells by introducing into the viral particle one of several specifically defined modifications. For example, an element which is recognized by the nuclear import machinery of a non-dividing cell can be associated with the nucleoprotein complex of the retrovirus. Alternatively, at least one protein encoded by viral gag or pol nucleic acid is modified so as to be recognized by the nuclear import machinery of a non-dividing cell.

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Retroviral Vectors Capable of Transducing Non-dividing Cells

FIELD OF THE INVENTION

The present invention relates generally to the field of viral vectors. In a specific aspect, the invention relates to novel recombinant retroviruses which are useful for the transfer and expression of nucleic acid sequences in non-dividing cells.

BACKGROUND OF THE INVENTION

The development of genetic vectors has heralded the fast-growing field of somatic gene transfer. Vectors based on simple retroviruses, such as the Moloney Leukemia Virus (MLV), are often selected because they efficiently integrate into the genome of the target cell. Integration is thought to be a prerequisite for long-term expression of the transduced gene. However, currently available retroviral vectors can only integrate in actively dividing cells. This feature severely limits the use of retroviral vectors for in vivo gene transfer. Non-dividing cells are the predominant, long-lived cell type in the body, and account for most desirable targets of gene transfer, including liver, muscle, and brain. Even protocols attempting the transduction of hematopoietic stem cells require demanding ex vivo procedures to trigger division of these cells prior to infection.

In the early steps of infection, retroviruses deliver their nucleoprotein core into the cytoplasm of the target cell. Here, reverse transcription of the viral genome takes place while the core matures into a preintegration complex. The complex must reach the nucleus to achieve integration of the viral DNA into the host cell chromosomes. For simple retroviruses (oncoretroviruses), this step requires the dissolution of the nuclear membrane at mitotic prophase, most likely because the bulky size of the preintegration complex prevents its passive diffusion through the nuclear pores.

While retroviral vectors are useful for many kinds of in vitro gene transfer studies, problems including low titers limit their use for some in vitro and most in vivo studies. Further, another problem is that integration of retroviral vectors into the host genome was thought to be restricted to cells undergoing DNA replication. Thus, although retroviral vectors capable of infecting a broad class of cell types are known, cell division is necessary for the proviral integration of these vectors. This effectively restricts the efficient use of retrovirus vectors to replicating cells. Thus, retroviruses have not been utilized to introduce genes into non-dividing or post-mitotic cells.

In contrast with oncoretroviruses, HIV and other lentiviruses have the ability to infect non-dividing cells, such as terminally differentiated macrophages and quiescent T lymphocytes. This property is likely crucial for HIV transmission, spread and persistence in the body, as well as for AIDS induction.

Matrix (MA) and Vpr govern the import of the HIV-1 nucleoprotein complex (NPC) through the nucleopore of the target cells in the absence of the breakdown of the nuclear envelope, allowing integration of the viral genome

into the host cell chromosome. MA has intrinsic karyophilic properties conferred by a highly conserved stretch of basic residues which acts as a nuclear localization signal (NLS). At the time of virus assembly, a subset of MA molecules undergo phosphorylation on their C-terminal tyrosine. Tyrosine-phosphorylated MA then binds to integrase (IN) and becomes a component of the NPC in which its karyophilic potential is revealed by interacting with one component of the cell nuclear import machinery, karyopherin α.

Vpr is another component of the viral NPC that exhibits karyophilic properties. However, Vpr does not contain a canonical NLS. Instead, the domain critical for nuclear localization of Vpr has recently been mapped to the two putative N-terminal α-helices thereof. Vpr could therefore play a crucial role in HIV-1 nuclear import through a pathway independent from MA; an NLS-independent pathway.

In corroboration of this hypothesis, it has been found that NLS peptide affects MA, but not Vpr, nuclear localization, and interferes with MA, but not Vpr-mediated HIV-1 nuclear import (see Gallay et al. in J. Virology 70:1027-1032 (1996)). The availability of these two distinct yet convergent pathways most probably ensures the ability of HIV-1 to infect non-dividing cells under a variety of conditions.

Mutant viruses lacking both MA NLS and Vpr cannot grow efficiently in terminally differentiated macrophages. In addition, an HIV-1-based vector containing the same mutations displayed lower in vivo levels of transduction of heterologous sequences in nonmitotic cells, such as neurons, compared with the wild-type vector, confirming the key roles of MA and Vpr in HIV-1 infection of non-dividing cells.

A retroviral vector system was recently derived from HIV (Naldini et al., Science 272:263-267 (1996)). In contrast to a murine leukemia virus-based counterpart, the HIV vector could transduce heterologous sequences into a variety of growth-arrested cells in vitro, as well as into human primary macrophages. Additionally, the HIV vector could mediate stable in vivo gene transfer into terminally differentiated neurons. The ability of HIV-based viral vectors to deliver genes in vivo into non-dividing cells could increase the applicability of retroviral vectors in gene therapy. However, a major limitation of these vectors is in their potential biohazard, as HIV is a major pathogen in humans.

Accordingly, there is still a need in the art for improved methods for the introduction of nucleic acids into non-dividing cells. In addition, retroviral constructs are needed which facilitate such methods.

BRIEF DESCRIPTION OF THE INVENTION

In accordance with the present invention, the above-described need in the art has been satisfied by the development of methods to modify retroviral vectors derived from viruses which are not known to be pathogenic in humans (e.g., MLV), so that such vectors are rendered capable of transducing heterologous sequences into non-dividing cells. Thus, it has been discovered that retroviruses can be rendered capable of infecting non-dividing cells by introducing into the viral particle one of several specifically defined modifications. For example, an element which is recognized by the nuclear import machinery of a non-dividing cell and which can associate with the nucleoprotein complex of the retrovirus can be introduced into a retrovirus.

In accordance with the present invention, it has been discovered that integrase (IN), the enzyme responsible for inserting the viral DNA into the host cell chromosomes, appears to play a dual role in HIV-1 infection of non-dividing cells. First, by binding to the C-terminal phosphotyrosine of matrix protein (MA), IN mediates the incorporation of the karyophilic properties of MA into the HIV-1 nucleoprotein complex (NPC). Second, IN facilitates the migration of the viral genome to the nucleopore by interacting with one component of the cell nuclear import machinery, karyopherin α (presumably through the IN NLS(s)). Thus, IN has been identified as a preferred "element" for use in the practice of the present invention.

In another embodiment of the present invention, at least one protein encoded by viral gag or pol nucleic acid is modified so as to be recognized by the nuclear import machinery of a non-dividing cell.

BRIEF DESCRIPTION OF THE FIGURE

Figure 1 illustrates the ability of HIV-1 integrase to enhance the transduction of non-dividing cells by an MLV vector.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided recombinant retroviruses capable of infecting non-dividing cell(s). Invention retroviruses comprise the following components:

a viral GAG,
a viral POL,
a viral ENV,
an element which associates with the nucleoprotein complex of said retrovirus, wherein said element is recognized by the

nuclear import machinery of said non-dividing cell,

an heterologous nucleic acid operably linked to a regulatory sequence, and

cis-acting nucleic acid necessary for reverse transcription and integration, and optionally, packaging of said retrovirus.

In accordance with another embodiment of the present invention, there are provided viral particles produced by the above-described recombinant retroviruses.

In accordance with yet another embodiment of the present invention, there are provided methods of producing the above-described recombinant retrovirus(es). Invention methods comprise transfecting a suitable packaging host cell with one or more vectors comprising:

a nucleic acid encoding a viral gag,

a nucleic acid encoding a viral pol,

a nucleic acid encoding a viral env,

a nucleic acid encoding an element which associates with the nucleoprotein complex of said retrovirus, wherein said element is recognized by the nuclear import machinery of said non-dividing cell, and

a nucleic acid encoding a packaging signal flanked by cis-acting nucleic acids necessary for reverse transcription and integration, and a cloning site for introduction of a heterologous nucleic acid, operably linked to a regulatory nucleic acid.

Optionally, invention method further comprises recovering the recombinant virus produced by the above-described transfected host cell.

Retroviruses are RNA viruses wherein the viral genome is RNA. When a host cell is infected with a retrovirus, the genomic RNA is reverse transcribed into a DNA intermediate which is integrated very efficiently into the chromosomal DNA of infected cells. This integrated DNA intermediate is referred to as a provirus. Transcription of the provirus and assembly into infectious virus occurs in a cell line containing appropriate sequences enabling encapsidation, or, if necessary, in the presence of an appropriate helper virus. A helper virus is not required for the production of the recombinant retrovirus of the present invention, since the sequences required for encapsidation are provided by co-transfection with appropriate vectors.

The retroviral genome and the proviral DNA have three genes: the gag, the pol, and the env, which are flanked by two long terminal repeat (LTR) sequences. The gag gene encodes the internal structural (matrix, capsid, and nucleocapsid) proteins; the pol gene encodes integrase and the RNA-directed DNA polymerase (reverse transcriptase) and the env gene encodes viral envelope glycoproteins. The 5' and 3' LTRs serve to promote transcription and polyadenylation of the virion RNAs. The LTRs contain all other cis-acting sequences necessary for viral replication. Lentiviruses have additional genes including vif, vpr, tat, rev, vpu, nef, and vpx (in HIV-1, HIV-2 and/or SIV).

Adjacent to the 5' LTR are sequences necessary for reverse transcription of the genome (the tRNA primer binding site) and for efficient encapsidation of viral RNA into particles (the Psi site). If the sequences necessary for encapsidation (or packaging of retrovirual RNA into infectious virions) are missing from the viral genome, the result is a cis defect which prevents encapsidation of genomic RNA. However, the resulting mutant is still capable of directing the synthesis of all virion proteins.

Invention retroviruses can be produced by a variety of methods, e.g., by transfecting a suitable host cell with one or more vectors comprising:

a nucleic acid encoding a viral gag;

a nucleic acid encoding a viral pol;

a nucleic acid encoding a viral env;

a nucleic acid encoding an element which associates with the nucleoprotein complex of said retrovirus, wherein said element is recognized by the nuclear import machinery of said non-dividing cell, and

a nucleic acid sequence encoding a packaging signal flanked by cis-acting nucleic acid sequences for reverse transcription and integration, and providing a cloning site for introduction of a heterologous gene, operably linked to a regulatory nucleic acid sequence.

A presently preferred method for the production of retroviruses according to the invention involves the use of a combination of a minimum of four vectors in order to produce a recombinant virion or recombinant retrovirus. A first vector provides a nucleic acid encoding a viral gag and a viral pol. These sequences encode a group specific antigen, reverse transcriptase, integrase and protease-enzymes necessary for reverse transcription, integration and maturation. Such sequences can be obtained from a variety of viral sources, e.g., Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), gibbon ape leukemia virus (GaLV), human immunodeficiency virus (HIV), Rous Sarcoma Virus (RSV), and the like.

A second vector employed in the practice of the present invention provides a nucleic acid encoding a viral envelope (env). The env gene can be derived from any virus, including retroviruses. The env may be amphotropic envelope protein which allows transduction of cells of human and other species, or may be ecotropic envelope

protein, which is able to transduce only mouse and rat cells. Further, it may be desirable to target the recombinant virus by linkage of the envelope protein with an antibody or a particular ligand for targeting to a receptor of a particular cell-type. By inserting a sequence (including regulatory region) of interest into the viral vector, along with another gene which encodes the ligand for a receptor on a specific target cell, for example, the vector is now target specific. Retroviral vectors can be made target specific by inserting, for example, a glycolipid, or a protein. Targeting is often accomplished by using an antibody to target the retroviral vector. Those of skill in the art will know of, or can readily ascertain without undue experimentation, specific methods to achieve delivery of a retroviral vector to a specific target.

Examples of retroviral-derived env genes include Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), gibbon ape leukemia virus (GaLV), human immunodeficiency virus (HIV), Rous Sarcoma Virus (RSV), and the like. Other env genes such as Vesicular stomatitis virus (VSV) (Protein G) can also be used.

The vector providing the viral env nucleic acid sequence is operably associated with regulatory sequence, e.g., a promoter or enhancer. Preferably, the regulatory sequence is a viral promoter. The regulatory sequence can be any eukaryotic promoter or enhancer, including for example, the Moloney murine leukemia virus promoter-enhancer element, the human cytomegalovirus enhancer (as used in the illustrative example), or the vaccinia P7.5 promoter. In some cases, such as the Moloney murine leukemia virus promoter-enhancer element, these promoter-enhancer elements are located within or adjacent to the LTR sequences.

A third vector contemplated for use in the practice of the present invention provides the cis-acting viral sequences necessary for the viral life cycle. Such sequences include the ψ packaging sequence, reverse transcription signals, integration signals, viral promoter, enhancer, and polyadenylation sequences. Such sequences can be obtained from a variety of viral sources, e.g., MoMuLV, HaMuSV, MuMTV, GaLV, HIV, RSV, and the like.

The third vector also contains a cloning site for introduction of a heterologous nucleic acid sequence. The cloning site (either containing the heterologous nucleic acid sequence therein, or absent any insert therein) can then be transferred to a non-dividing cell. The heterologous nucleic acid sequence is typically incorporated into this vector prior to incorporation of products encoded thereby into a viral particle. Thus, for example, this third vector can be used for the preparation of a viral particle containing the heterologous nucleic acid sequence, thereby facilitating the direct introduction of the heterologous nucleic acid sequence into a non-dividing cell.

A fourth vector contemplated for use in the practice of the present invention provides the element which associates with the nucleoprotein complex of said retrovirus. For example, as illustrated in Example 8, HIV-1 integrase is expressed under the control of the HIV-1 promoter.

Since recombinant retroviruses produced by standard methods in the art are defective, they require assistance in order to produce infectious vector particles. Typically, this assistance is provided, for example, by using a helper cell line that provides the missing viral functions. These plasmids are missing a nucleotide sequence which enables the packaging mechanism to recognize

an RNA transcript for encapsidation. Examples of helper cell lines which have deletions of the packaging signal include Ψ2, PA317, PA12, and the like. Suitable cell lines produce empty virions, since no genome is packaged. If a retroviral vector is introduced into such cells in which the packaging signal is intact, but the structural genes are replaced by other genes of interest, the vector can be packaged and vector virion produced.

The invention method for production of recombinant retrovirus employs different constructs than are employed in the standard helper virus/packaging cell line method described above. The four or more individual vectors used to co-transfect a suitable packaging cell line collectively contain all of the required genes for production of a recombinant virus for infection and transfer of nucleic acid to a non-dividing cell. Consequently, there is no need for use of a helper virus.

As readily recognized by those of skill in the art, a variety of different packaging cell lines can be prepared in accordance with the present invention. Thus, for example, a stable packaging cell line containing several of the above-described vectors can be prepared, such that one only need introduce a vector containing the heterologous nucleic acid sequence in order to produce a virion which is capable of infecting non-dividing cells and hence delivering heterologous nucleic acid sequences thereto.

Thus, in accordance with another embodiment of the invention, there are provided stable packaging cell lines containing:

a nucleic acid encoding a viral gag,
a nucleic acid encoding a viral pol,
a nucleic acid encoding a viral env, and

a nucleic acid encoding an element which associates with the nucleoprotein complex of a retrovirus, wherein said element is recognized by the nuclear import machinery of a non-dividing cell.

The above-described cell lines are especially useful for the rapid introduction of heterologous nucleic acid sequences into a host. Thus, a vector encoding:

a nucleic acid encoding a packaging signal flanked by cis-acting nucleic acids necessary for reverse transcription and integration, and a heterologous nucleic acid operably linked to a regulatory nucleic acid

is introduced into the above-described stable packaging cell line, which is then induced to produce viral particles. The resulting viral particles are useful for the introduction of heterologous nucleic acid sequences into non-dividing cells.

In accordance with yet another embodiment of the invention, there are provided stable packaging cell lines containing:

a first nucleic acid encoding a viral gag,
a second nucleic acid encoding a viral pol, wherein at least one protein encoded by said first or second nucleic acid is modified so as to be recognized by the nuclear import machinery of a non-dividing cell, and
a nucleic acid encoding a viral env.

The above-described cell lines are similarly useful for the rapid introduction of heterologous nucleic acid sequences into a host, as described hereinabove.

Elements which associate with the nucleoprotein complex of a retrovirus (either directly or indirectly), and which are recognized by the nuclear import machinery of a non-dividing cell, as contemplated for use in the practice of the present invention include viral proteins which are directly recognized by the nuclear import machinery of a non-dividing cell, such as, for example, matrix protein (MA), integrase (IN), and the like, as well as viral proteins which are indirectly recognized by the nuclear import machinery of a non-dividing cell (by associating with the nucleoprotein complex and an agent which is recognized by the nuclear import machinery of a non-dividing cell), such as, for example, reverse transcriptase (RT), nucleocapsid, protease, and the like. A presently preferred viral protein contemplated for use herein is a lentiviral integrase, with HIV integrase being particularly preferred.

Additional elements contemplated for use in the practice of the present invention include nuclear localization signals, wherein said NLS is operably associated with the nucleoprotein complex of a retrovirus. Those of skill in the art can readily identify NLSs suitable for use herein. See, for example, the numerous NLS sequences described by Dingwall and Laskey in TIBS 16:478-481 (1991) and Goerlich and Mattaj in Science 271:1513-1518 (1996). For example, a suitable NLS can be derived from HIV-1 integrase. Another protein that exhibits karyophilic properties, and hence is useful herein, is Vpr. In addition, consensus NLSs have also been identified in the art, characterized as comprising a contiguous sequence of seventeen amino acids, wherein the first two amino acids are basic amino acids, followed by a spacer region of any ten amino acids, followed by a basic cluster in which at least three of the next five amino acids are basic.

In addition, numerous specific NLSs have been identified in the art, e.g.:

the amino acid sequence KRKQ (SEQ ID NO:1),
the amino acid sequence KELQKQ (SEQ ID NO:2),
the amino acid sequence KRKGGIG (SEQ ID NO:3),
the amino acid sequence PKKKRKV (SEQ ID NO:4),
the amino acid sequence AAFEDLRVLS (SEQ ID NO:5),
the yeast GAL4 targeting signal,
and the like.

Heterologous nucleic acid sequence(s) employed in the practice of the present invention are operably linked to a regulatory nucleic acid sequence. As used herein, the term "heterologous" nucleic acid sequence refers to a sequence that originates from a foreign species, or, if from the same species, it may be substantially modified from its original form. Alternatively, an unchanged nucleic acid sequence that is not normally expressed in a cell is a heterologous nucleic acid sequence. As used herein, the term "operably linked" refers to a functional linkage between the regulatory sequence and the heterologous nucleic acid sequence. Preferably, the heterologous sequence is linked to a promoter, resulting in a chimeric gene. The heterologous nucleic acid sequence is preferably under control of either the viral LTR promoter-enhancer signals or of an internal promoter. Retained signals within the retrovirual LTR can still bring about efficient integration of the vector into the host cell genome.

The promoter sequence may be homologous or heterologous to the desired gene sequence. A wide range of

promoters are suitable for use in the practice of the present invention, including viral or mammalian promoters. Cell or tissue specific promoters can be utilized to target expression of gene sequences in specific cell populations. Mammalian and viral promoters suitable for use in the practice of the present invention are well knwn and readily available in the art.

Preferably during the cloning stage, the nucleic acid construct referred to as the transfer vector, having the packaging signal and the heterologous cloning site, will also contain a selectable marker gene. Marker genes are utilized to assay for the presence of the vector, and thus, to confirm infection and integration. The presence of this marker gene ensures the growth of only those host cells which express the inserted DNA. Typical selection genes encode proteins that confer resistance to antibiotics and other toxic substances, e.g., histidinol, puromycin, hygromycin, neomycin, methotrexate, and the like. Other marker systems commmonly used in the art include ß-galactosidase (LacZ) and luciferase reporter or marker systems, which can conveniently be monitored visually.

Recombinant viruses of the invention are capable of transferring nucleic acid sequence(s) into non-dividing cells. The term nucleic acid sequence refers to any nucleic acid molecule, preferably DNA. The nucleic acid molecule may be derived from a variety of sources, including DNA, cDNA, synthetic DNA, RNA, or combinations thereof. Such nucleic acid sequences may comprise genomic DNA which may or may not include naturally occurring introns. Moreover, such genomic DNA may be obtained in association with promoter regions, introns, or poly A sequences. Genomic DNA may be extracted and purified from suitable cells by means well known in the art. Alternatively, messenger RNA (mRNA) can be isolated from

cells and used to produce cDNA by reverse transcription or other means.

The phrase "non-dividing" cell refers to a cell that does not go through mitosis. Non-dividing cells may be blocked at any point in the cell cycle, (e.g., G_0/G_1, G_1/S, G_2/M), as long as the cell is not actively dividing. For ex vivo infection, a dividing cell can be treated to block cell division by standard techniques used by those of skill in the art, such as, for example, irradiation, aphidocolin treatment, serum starvation, contact inhibition, and the like. However, it should be understood that ex vivo infection is often performed without blocking the cells since many cells are already arrested (e.g., stem cells).

Recombinant retrovirus vectors according to the present invention are capable of infecting any non-dividing cell, regardless of the mechanism used to block cell division or the point in the cell cycle at which the cell is blocked. Examples of pre-existing non-dividing cells in the body include neurons,. myocytes, hepatocytes, hematopoietic stem cells, lymphocytes, dendritic cells, epithelial cells, macrophages, and the like, as well as derivatives thereof. Recombinant retrovirus vectors according to the present invention can be used for both in vivo gene delivery (for example, by injection), as well as ex vivo gene delivery.

The method of the present invention contemplates the use of at least three vectors which provide all of the functions required for packaging of recombinant virions as discussed above, plus a fourth vector which provides the element which is capable of associating with the nucleoprotien complex, thereby facilitating infection of non-dividing cells. Invention method also contemplates transfection of vectors including viral genes such as vpr,

vif, nef, vpx, tat, rev, and vpu. Some or all of these genes can be included, for example, on the packaging construct vector, or, alternatively, they may reside on individual vectors. There is no limitation to the number of vectors which can be utilized, as long as they are co-transfected to the packaging cell line in order to produce a single recombinant retrovirus. For example, one could put the env nucleic acid sequence on the same construct as the gag and pol.

Viral vectors contemplated for use herein are introduced via transfection or infection into a packaging cell line. The packaging cell line produces viral particles that contain the vector genome. Methods for transfection or infection are well known by those of skill in the art. After co-transfection of the at least four vectors to the packaging cell line, the recombinant virus is recovered from the culture media and titered by standard methods used by those of skill in the art.

In accordance with yet another aspect of the present invention, there is provided another class of recombinant retroviruses which are capable of infecting non-dividing cell(s). This class of invention retroviruses comprises the following components:

a viral GAG,

a viral POL,

a viral ENV,

an heterologous nucleic acid operably linked to a regulatory sequence, and

cis-acting nucleic acid necessary for reverse transcription and integration,

wherein a protein associated with the nucleoprotein complex of said retrovirus is modified so as to be recognized by the nuclear import machinery of said non-dividing cell.

In accordance with another embodiment of the present invention, there are provided viral particles produced by the above-described recombinant retroviruses.

In accordance with still another embodiment of the present invention, there are provided methods of producing the above-described recombinant retrovirus(es). According to this embodiment of the invention, invention infection method comprises transfecting a suitable packaging host cell with one or more vectors comprising:

a first nucleic acid encoding a viral gag,

a second nucleic acid encoding a viral pol, wherein at least one protein encoded by said first or second nucleic acid is modified so as to be recognized by the nuclear import machinery of said non-dividing cell,

a nucleic acid encoding a viral env, and

a nucleic acid encoding a packaging signal flanked by cis-acting nucleic acids necessary for reverse transcription and integration, and a cloning site for introduction of a heterologous nucleic acid, operably linked to a regulatory nucleic acid.

Optionally, invention method further comprises recovering the recombinant virus produced by the above-described transfected host cell.

A variety of modifications of proteins associated with the nucleoprotein complex of said retrovirus are contemplated for use herein, such as, for example, by mutation of the protein associated with the nucleoprotein complex of said retrovirus so as to be recognized by the nuclear import machinery of said non-dividing cell. Alternatively, the protein associated with the

nucleoprotein complex of the retrovirus can be modified by the addition of a karyophilic agent thereto.

Examplary karyophilic agents contemplated for use herein include reverse transcriptase, matrix protein, nucleocapsid, protease, integrase, and the like.

As yet another alternative, retrovirus according to the invention can be prepared employing protein associated with the nucleoprotein complex thereof which has been modified by the addition thereto of a nuclear localization signal, as described hereinabove.

In accordance with yet another embodiment of the present invention, there are provided methods for the introduction and expression of heterologous nucleic acids in non-dividing cell(s). Invention methods comprise infecting non-dividing cell(s) with any of the recombinant viruses described herein, and expressing the heterologous nucleic acid in said non-dividing cell.

It may be desirable to modulate the expression of a gene regulating molecule in a cell by the introduction of a molecule by the method of the invention. The term "modulate" envisions the suppression of expression of a gene when it is over-expressed, or augmentation of expression when it is under-expressed. Where a cell proliferative disorder is associated with the expression of a gene, nucleic acid sequences that interfere with the gene's expression at the translational level can be used. This approach utilizes, for example, antisense nucleic acid, ribozymes, or triplex agents to block transcription or translation of a specific mRNA, either by masking that mRNA with an antisense nucleic acid or triplex agent, or by cleaving it with a ribozyme.

Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule (Weintraub, Scientific American, 262:40, 1990). In the cell, the antisense nucleic acids hybridize to the corresponding mRNA, forming a double-stranded molecule. The antisense nucleic acids interfere with the translation of the mRNA, since the cell will not translate a mRNA that is double-stranded. Antisense oligomers of about 15 nucleotides are preferred, since they are easily synthesized and are less likely to cause problems than larger molecules when introduced into the target cell. The use of antisense methods to inhibit the in vitro translation of genes is well known in the art (Marcus-Sakura, Anal.Biochem., 172:289, 1988).

The antisense nucleic acid can be used to block expression of a mutant protein or a dominantly active gene product, such as amyloid precursor protein that accumulates in Alzheimer's disease. Such methods are also useful for the treatment of Huntington's disease, hereditary Parkinsonism, and other diseases. Antisense nucleic acids are also useful for the inhibition of expression of proteins associated with toxicity.

Use of an oligonucleotide to stall transcription is known as the triplex strategy since the oligomer winds around double-helical DNA, forming a three-strand helix. Therefore, these triplex compounds can be designed to recognize a unique site on a chosen gene (Maher, et al., Antisense Res. and Dev., 1(3):227, 1991; Helene, C., Anticancer Drug Design, 6(6):569, 1991).

Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA in a manner analogous to DNA restriction endonucleases. Through the modification of nucleotide sequences which encode these RNAs, it is possible to engineer molecules

that recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech, J. Amer. Med. Assn., 260:3030, 1988). A major advantage of this approach is that, because they are sequence-specific, only mRNAs with particular sequences are inactivated.

It may be desirable to transfer a nucleic acid encoding a biological response modifier. Included in this category are immunopotentiating agents including nucleic acids encoding a number of the cytokines classified as "interleukins". These include, for example, interleukins 1 through 12. Also included in this category, although not necessarily working according to the same mechanisms, are interferons, and in particular gamma interferon (γ-IFN), tumor necrosis factor (TNF) and granulocyte-macrophage-colony stimulating factor (GM-CSF). It may be desirable to deliver such nucleic acids to bone marrow cells or macrophages to treat enzymatic deficiencies or immune defects. Nucleic acids encoding growth factors, toxic peptides, ligands, receptors, or other physiologically important proteins can also be introduced into specific non-dividing cells.

The recombinant retrovirus of the invention can be used to treat an HIV infected cell (e.g., T-cell or macrophage) with an anti-HIV molecule. In addition, respiratory epithelium, for example, can be infected with a recombinant retrovirus of the invention having a gene for cystic fibrosis transmembrane conductance regulator (CFTR) for treatment of cystic fibrosis.

The method of the invention may also be useful for neuronal or glial cell transplantation, or "grafting", which involves transplantation of cells infected with the recombinant retrovirus of the invention ex vivo, or infection in vivo into the central nervous system or into the ventricular cavities or subdurally onto the surface of

a host brain. Such methods for grafting will be known to those skilled in the art and are described in Neural Grafting in the Mammalian CNS, Bjorklund and Stenevi, eds., (1985), incorporated by reference herein. Procedures include intraparenchymal transplantation, (i.e., within the host brain) achieved by injection or deposition of tissue within the host brain so as to be apposed to the brain parenchyma at the time of transplantation.

Administration of the cells or virus into selected regions of the recipient subject's brain may be made by drilling a hole and piercing the dura to permit the needle of a microsyringe to be inserted. The cells or recombinant retrovirus can alternatively be injected intrathecally into the spinal cord region. A cell preparation infected ex vivo, or the recombinant retrovirus of the invention, permits grafting of neuronal cells to any predetermined site in the brain or spinal cord, and allows multiple grafting simultaneously in several different sites using the same cell suspension or viral suspension and permits mixtures of cells from different anatomical regions.

Cells infected with a recombinant retrovirus of the invention, in vivo, or ex vivo, used for treatment of a neuronal disorder for example, may optionally contain an exogenous gene, for example, a gene which encodes a receptor or a gene which encodes a ligand. Such receptors include receptors which respond to dopamine, GABA, adrenaline, noradrenaline, serotonin, glutamate, acetylcholine and other neuropeptides, as described above. Examples of ligands which may provide a therapeutic effect in a neuronal disorder include dopamine, adrenaline, noradrenaline, acetylcholine, gamma-aminobutyric acid and serotonin. The diffusion and uptake of a required ligand after secretion by an infected donor cell would be beneficial in a disorder where the subject's neural cell is

defective in the production of such a gene product. A cell genetically modified to secrete a neurotrophic factor, such as nerve growth factor (NGF), might be used to prevent degeneration of cholinergic neurons that might otherwise die without treatment. Alternatively, cells be grafted into a subject with a disorder of the basal ganglia, such as Parkinson's disease, can be modified to contain an exogenous gene encoding L-DOPA, the precursor to dopamine. Parkinson's disease is characterized by a loss of dopamine neurons in the substantia-nigra of the midbrain, which have the basal ganglia as their major target organ.

Other neuronal disorders that can similarly be treated according to the method of the invention include Alzheimer's disease, Huntington's disease, neuronal damage due to stroke, damage in the spinal cord, and the like. Alzheimer's disease is characterized by degeneration of the cholinergic neurons of the basal forebrain. The neurotransmitter for these neurons is acetylcholine, which is necessary for their survival. Engraftment of cholinergic cells infected with a recombinant retrovirus of the invention containing an exogenous gene for a factor which would promote survival of these neurons can be accomplished by the method of the invention. Following a stroke, there is selective loss of cells in the CA1 of the hippocampus as well as cortical cell loss which may underlie cognitive function and memory loss in these patients. Once identified, molecules responsible for CA1 cell death can be inhibited by the methods of this invention. For example, antisense sequences, or a gene encoding an antagonist can be transferred to a neuronal cell and implanted into the hippocampal region of the brain.

The method of transferring nucleic acid also contemplates the grafting of neuroblasts in combination with other therapeutic procedures useful in the treatment

of disorders of the CNS. For example, the retroviral infected cells can be co-administered with agents such as growth factors, gangliosides, antibiotics, neurotransmitters, neurohormones, toxins, neurite promoting molecules and antimetabolites and precursors of these molecules such as the precursor of dopamine, L-DOPA.

Further, there are a number of inherited neurologic diseases in which defective genes may be replaced including: lysosomal storage diseases such as those involving ß-hexosaminidase or glucocerebrosidase; deficiencies in hypoxanthine phosphoribosyl transferase activity (the "Lesch-Nyhan" syndrome); amyloid polyneuropathies (prealbumin); Duchenne's muscular dystrophy, and retinoblastoma, for example.

For diseases due to deficiency of a protein product, gene transfer could introduce a normal gene into the affected tissues for replacement therapy, as well as to create animal models for the disease using antisense mutations. For example, it may be desirable to insert a Factor IX encoding nucleic acid into a retrovirus for infection of a muscle or liver cell.

The invention will now be described in greater detail by reference to the following non-limiting examples.

Example 1

Integrase (IN) contains multiple putative nuclear localization signals (NLSs). To determine the role of IN in nuclear import of the HIV-1 nucleoprotein complex (NPC), an HIV-1 mutant lacking IN, called ΔIN, was generated by introducing a stop codon at the 3' end of the reverse transcriptase (RT) coding sequence and then used to acutely infect irradiated HeLa cells. At 1 hour and 8 hours post-infection, cytoplasmic and nuclear extracts were analyzed

for the presence of nucleocapsid (NC) and RT, other components of the NPC. In wild-type (wt) infected cells, NC or RT were predominantly associated with the cytoplasmic compartment at 1 hour, whereas they were found in the nucleus at 8 hours (about 50% of molecules).

Similar results were observed using strains defective for IN (ΔIN) or mutated in both Vpr and the MA NLS (ΔNLS Δvpr). In contrast, no nuclear translocation of NC or of RT was observed for a strain defective for all three determinants (MA ΔNLS Δvpr ΔIN). As control, the subcellular localization of HIV-1 capsid (CA) was monitored. CA remains associated with the cytoplasmic compartment. Altogether, these findings strongly suggest that IN is capable of functioning as a mediator of HIV-1 nuclear import in non-dividing cells.

Example 2
IN is a karyophilic protein

To investigate whether IN contains karyophilic properties, cells were transfected with a construct expressing IN alone. Nuclear and cytoplasmic extracts were then analyzed for their IN content. IN was found essentially associated with the nucleus of transfected cells. In addition, fluorescein-isothiocyanate (FITC)-labeled IN microinjected in the cytoplasm of cells was also detected predominantly in the nucleus of these cells, whereas FITC-BSA used as control remained associated with the cytoplasm.

These results demonstrate that IN is a karyophilic protein.

Example 3

IN as a component of HIV-1 NPC interacts with cell nuclear import machinery

The interaction of IN with the cell nuclear import machinery was then investigated. To explore this issue, purified HIV-1 NPC were immunoprecipitated with anti-karyopherin α antibodies, and the bound material was then analyzed by immunoblot using anti-IN, anti-MA antibodies as detectors. Interestingly, in HIV-1 NPC extracted with mild conditions (0.05% of detergent), IN co-immunoprecipitated with karyopherin α. Under the same conditions, MA also co-immunoprecipitated. Since MA and IN are associated in the HIV-1 pre-integration complex (PIC), it could not be ascertained that IN did not indirectly bind karyopherin α, through MA. However, when HIV-1 PIC were extracted with stringent conditions (0.5% of detergent), IN (but not MA) co-immunoprecipitated with karyopherin α, suggesting a direct association between the two molecules.

Example 4

IN directly binds karyopherin α in an NLS-dependent manner

The binding of karyopherin α with IN was then investigated in vitro. Recombinant GST-IN fusion protein could capture one member of the karyopherin α family (karyopherin α-2), called Rch1.1, overexpressed in transfected cells. In contrast, GST alone, GST-CA or GST-Nef could not bind Rch1.1. Furthermore, NLS peptide blocked IN-Rch1.1 complex formation, whereas reverse peptide has no effect. In addition, a GST-recombinant form of another member of the karyopherin α family (karyopherin α-1), called hSRP1, could bind recombinant IN. These data demonstrate the NLS-dependent direct binding of IN to karyopherin α.

Example 5

The C-terminal domain of IN contains two NLSs recognized by karyopherin

To map the IN domains recognized by karyopherin α, truncated forms of GST-IN were generated and tested for their affinity to Rch1.1. Rch1 was captured by a GST-IN fusion protein lacking 50 N-terminal amino acids of IN (50-288) but not by variants deleted at the C-terminus (1-170 or 50-185). From these results, it could be deduced that the IN domain recognized by karyopherin α is located between residues 185 and 288. In this region, two putative NLS were noted; NLS_1, located around positions 186-188 (KRKQ) and NLS_2, around positions 211-216 (KELQKQ). Both NLSs were mutated and the resulting GST-ΔNLS IN proteins were tested for their affinity for Rch1.1. Mutations introduced in NLS_1 completely abrogated the IN-Rch1 complex formation, whereas mutations in NLS_2 decreased (about 50%) the interaction between the two moles. These data suggest that NLS_1 IN is the main site of recognition of karyopherin α, although NLS_2 IN appears to contribute to the stability of this interaction. Interestingly, NLS_1 IN (consensus: KRKGGIG) is highly conserved among lentiviruses (HIV-1, HIV-2, SIV, BIV, FIV, Visna, CAEV, EIAV), but is absent in non-lentiviruses (MLV, RSV, HTLV), suggesting an important role for this NLS motif in lentiviral infection.

To confirm the previous results, FITC-labeled GST-$ΔNLS_1$ or GST-$ΔNLS_2$ were microinjected in the cytoplasm of cells and their subcellular localization was monitored by confocal microscopy. Both NLS mutants did not enter inside the nucleus, but instead remained associated with the cytoplasmic compartment. These findings indicate that two IN C-terminal NLSs are likely involved in IN-κα complex formation and IN nuclear import.

Example 6
Recruitment of karyopherin ß and nucleoporin by IN-κα complexes in vitro

The events involved in IN-mediated HIV-1 nuclear import were recapitulated in vitro. Karyopherin α and karyopherin ß were successively added to GST-IN to mimic the sequential docking of IN to the nucleopore. Karyopherin ß bound to GST-IN-karyopherin α complexes but not to GST-IN alone. No binding of components of the cell nuclear import machinery was observed when GST alone was used as negative control. In addition, neither of the NLS IN mutants (i.e., GST-ΔNLS1 IN and GST-ΔNLS2 IN) recruited karyopherin α (or any of the other components of the cell nuclear import machinery). These findings corroborate the model of nuclear import of NLS-bearing protein, in which an NLS-substrate, such as IN or MA, is first recognized by the cytosolic karyopherin α through its NLS, and then docked to the nucleopore via karyopherin ß.

Example 7
IN governs HIV-1 nuclear import in γ-irradiated CD4+ HeLa cells through its NLSs

To determine the role of the karyophilic motifs of IN in HIV-1 infection of non-dividing cells, γ-irradiated CD4+ HeLa cells were acutely infected with a vpr-defective strain mutated in both MA NLS and IN NLS_1 (Δvpr MA ΔNLS IN ΔNLS1). As previously, at 1 hour and 8 hours post-infection, cytoplasmic and nuclear extracts were analyzed for the presence of components of HIV-1 NPC; NC and RT. Importantly, no nuclear translocation of NC or of RT was observed 8 hours post-infection for a strain defective for the three karyophilic viral determinants; the IN NLS_1, the MA NLS and Vpr (MA ΔNLS Δvpr IN ΔNLS_1). These findings strongly suggest that IN, through its NLS, is a

main mediator of HIV-1 nuclear import in γ-irradiated CD4+ HeLa cells.

Example 8

HIV-1 Integrase induces infection of non-dividing cells by MLV-based vectors

Human HeLa cells were arrested in G1-S stage by aphidicolin treatment, then infected with:

- an MLV-based vector (encoding the marker, beta-galactosidase), pseudotyped with the VSV G envelope,
- an HIV-based vector (encoding the marker, beta-galactosidase), pseudotyped with the VSV G envelope, or
- a "chimeric" vector according to the invention, wherein the above-described MLV-based vector is supplemented with an additional vector encoding HIV-1 integrase.

For comparison, non-arrested (i.e., dividing) HeLa cells were infected with each of the above-described vectors.

Transduction was scored by X-gal staining of the cultures 48 hours after infection. Results are presented in Figure 1, and are expressed as the number of blue cells per 50 μl of supernatant.

Inspection of Figure 1 reveals that the HIV-based vector is capable of infecting non-dividing cells as well as dividing cells. In contrast, while the MLV-based vector is capable of infecting dividing cells, this vector is virtually incapable of infecting arrested cells. However, infection of non-dividing cells is surprisingly effective employing a chimeric construct according to the invention.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Trono, Didier P.
Gallay, Philippe A.

(ii) TITLE OF THE INVENTION: RETROVIRAL VECTORS CAPABLE OF TRANSDUCING NON-DIVIDING CELLS

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows DEMONSTRATION Version 2.0D

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: 08/715,318
(B) FILING DATE: 17-SEP-1996
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
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(B) REGISTRATION NUMBER: 31,192
(C) REFERENCE/DOCKET NUMBER: P41 90295

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 619-677-1409
(B) TELEFAX: 619-677-1465
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Lys Arg Lys Gln
1

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Lys Glu Leu Gln Lys Gln
1 5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Lys Arg Lys Gly Gly Ile Gly
1 5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Pro Lys Lys Lys Arg Lys Val
1 5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ala Ala Phe Glu Asp Leu Arg Val Leu Ser
1 5 10

That which is claimed is:

1. A recombinant retrovirus capable of infecting a non-dividing cell, said retrovirus comprising the following components:

a viral GAG,

a viral POL,

a viral ENV,

an element which associates with the nucleoprotein complex of said retrovirus, wherein said element is recognized by the nuclear import machinery of said non-dividing cell,

an heterologous nucleic acid operably linked to a regulatory sequence, and

cis-acting nucleic acid necessary for reverse transcription and integration.

2. A retrovirus according to claim 1 wherein said element is selected from reverse transcriptase, matrix protein, nucleocapsid, protease or integrase.

3. A retrovirus according to claim 2 wherein said element is a lentiviral integrase.

4. A retrovirus according to claim 3 wherein said element is HIV integrase.

5. A retrovirus according to claim 1 wherein said element is a nuclear localization signal, operably associated with the nucleoprotein complex of said retrovirus.

6. A retrovirus according to claim 5 wherein said nuclear localization signal is derived from HIV-1 integrase.

7. A retrovirus according to claim 1 wherein the components of said retrovirus are selected based on the type of non-dividing cell targeted for introduction of said heterologous nucleic acid.

8. A retrovirus according to claim 7 wherein the components of said retrovirus are obtained from MoMuLV, HaMuSV, MuMTV, GaLV, HIV or RSV.

9. A retrovirus according to claim 7 wherein said non-dividing cell is a neuron, a myocyte, an hepatocyte, an hematopoietic stem cell, a lymphocyte, a dendritic cell, an epithelial cell or a macrophage.

10. A viral particle produced by the recombinant retrovirus of claim 1.

11. A recombinant retrovirus capable of infecting a non-dividing cell, said retrovirus comprising the following components:

a viral GAG,

a viral POL,

a viral ENV,

an heterologous nucleic acid operably linked to a regulatory sequence, and

cis-acting nucleic acid necessary for reverse transcription and integration,

wherein a protein associated with the nucleoprotein complex of said retrovirus is modified so as to be recognized by the nuclear import machinery of said non-dividing cell.

12. A retrovirus according to claim 11 wherein said protein associated with the nucleoprotein complex of said retrovirus is mutated so as to be recognized by the nuclear import machinery of said non-dividing cell.

13. A retrovirus according to claim 11 wherein said protein associated with the nucleoprotein complex of said retrovirus is modified by the addition of a karyophilic agent thereto.

14. A retrovirus according to claim 13 wherein said karyophilic agent is matrix protein or integrase.

15. A retrovirus according to claim 13 wherein said karyophilic agent interacts with said nucleoprotein complex by association with reverse transcriptase, nucleocapsid or protease.

16. A retrovirus according to claim 11 wherein said protein associated with the nucleoprotein complex of said retrovirus is modified by the addition thereto of a nuclear localization signal.

17. A retrovirus according to claim 11 wherein the components of said retrovirus are selected based on the type of non-dividing cell targeted for introduction of said heterologous nucleic acid.

18. A retrovirus according to claim 17 wherein the components of said retrovirus are obtained from MoMuLV, HaMuSV, MuMTV, GaLV, HIV or RSV.

19. A retrovirus according to claim 17 wherein said non-dividing cell is a neuron, a myocyte, an hepatocyte, an hematopoietic stem cell, a lymphocyte, a dendritic cell, an epithelial cell or a macrophage.

20. A viral particle produced by the recombinant retrovirus of claim 11.

21. A method of producing a recombinant retrovirus capable of infecting a non-dividing cell, said method comprising transfecting a suitable packaging host cell with one or more vectors comprising:

a nucleic acid encoding a viral gag,

a nucleic acid encoding a viral pol,

a nucleic acid encoding a viral env,

a nucleic acid encoding an element which associates with the nucleoprotein complex of said retrovirus, wherein said element is recognized by the nuclear import machinery of said non-dividing cell, and

a nucleic acid encoding a packaging signal flanked by cis-acting nucleic acids necessary for reverse transcription and integration, and a cloning site for introduction of a heterologous nucleic acid, operably linked to a regulatory nucleic acid.

22. A method according to claim 21 further comprising recovering the recombinant virus produced by said transfected host cell.

23. A method of producing a recombinant retrovirus capable of infecting a non-dividing cell, said method comprising transfecting a suitable packaging host cell with one or more vectors comprising:

a first nucleic acid encoding a viral gag,

a second nucleic acid encoding a viral pol, wherein at least one protein encoded by said first or second nucleic acid is modified so as to be recognized by the nuclear import machinery of said non-dividing cell,

a nucleic acid encoding a viral env, and

a nucleic acid encoding a packaging signal flanked by cis-acting nucleic acids necessary for reverse transcription and integration, and a cloning site for introduction of a heterologous nucleic acid, operably linked to a regulatory nucleic acid.

24. A method according to claim 23 further comprising recovering the recombinant virus produced by said transfected host cell.

25. A method for the introduction and expression of a heterologous nucleic acid in a non-dividing cell, said method comprising infecting said non-dividing cell with a recombinant virus according to claim 1, and expressing the heterologous nucleic acid in said non-dividing cell.

26. A method for the introduction and expression of a heterologous nucleic acid in a non-dividing cell, said method comprising infecting said non-dividing cell with a recombinant virus according to claim 11, and expressing the heterologous nucleic acid in said non-dividing cell.

27. A stable packaging cell line containing:
a nucleic acid encoding a viral gag,
a nucleic acid encoding a viral pol,
a nucleic acid encoding a viral env, and
a nucleic acid encoding an element which associates with the nucleoprotein complex of a retrovirus, wherein said element is recognized by the nuclear import machinery of a non-dividing cell.

28. A stable packaging cell line containing:
a first nucleic acid encoding a viral gag,
a second nucleic acid encoding a viral pol, wherein at least one protein encoded by said first or second nucleic acid is modified so as to be recognized by the nuclear import machinery of said non-dividing cell, and
a nucleic acid encoding a viral env.

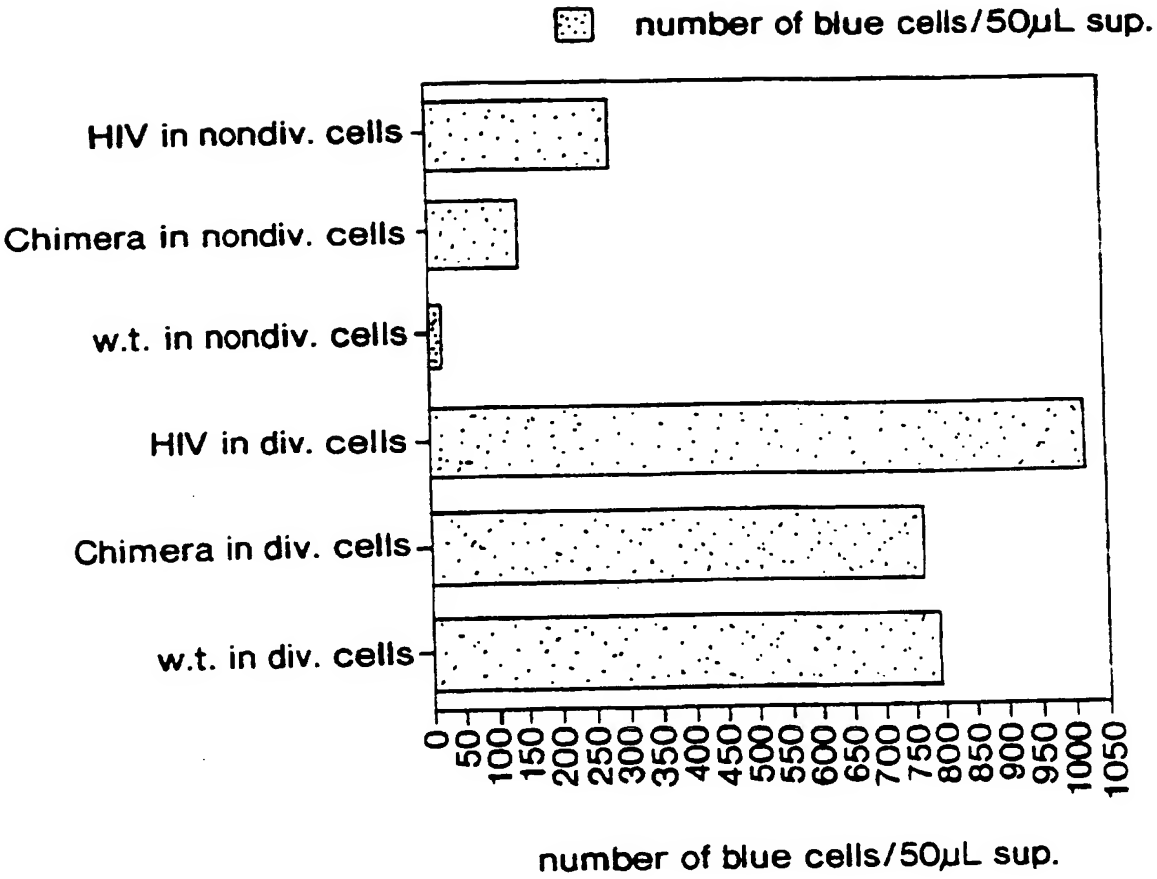

FIG.1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/15934

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :C12N 15/09, 7/01, 7/02
US CL :435/172.3, 235.1, 239
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3, 235.1, 239, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, CAPLUS, EMBASE, WPIDS, SCISEARCH
search terms: retrovir?, nondividing cell#

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BUKRINSKY, et al. A Nuclear Localization Signal within HIV-1 Matrix Protein that Governs Infection of Non-Dividing Cells. Nature. 14 October 1993, Vol. 365, pages 666-669. See especially pages 667-668.	1-6, 11, 13-16, 20-22
X	NALDINI et al. In Vivo Gene Delivery and Stable Transduction of Nondividing Cells by a Lentiviral Vector. Science. 12 April 1996, Vol. 272, pages 263-267. See especially pages 263 and 265.	1-6, 10, 21-22

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 29 OCTOBER 1997	Date of mailing of the international search report 24 NOV 1997
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